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(12) Patent: (11) CA 1297816
Application Number: (21) 509243

(54) PLATELET FUNCTION INHIBITING MONOCLONAL ANTIBODY FRAGMENT

(54) FRAGMENT D'ANTICORPS MONOCLONAL INHIBANT LA FONCTION PLAQUETTAIRE

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Patent Cooperation Treaty (PCT): No

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851,711	United States	Apr. 14, 1986
621,378	United States	June 14, 1985

Availability of licence:	N/A
Language of filing:	English

ABSTRACT:

ABSTRACT OF THE DISCLOSURE
There is provided a novel monoclonal antibody fragment which inhibits platelet function and may be useful in the treatment of thrombotic diseases. The fragment is produced by proteolytic digestion of the 7E3 monoclonal antibody.
SUNY109 Suny119ER
#5

CLAIMS: Show all claims

*** Note: Data on abstracts and claims is shown in the official language in which it was submitted.

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WHAT IS CLAIMED IS:

1. A fragment of a monoclonal antibody of the class IgG_1 produced by a hybridoma formed by fusion of cells from a mouse myeloma line and spleen cells from a mouse previously immunized with human blood platelets, which antibody fragment inhibits platelet function and thrombosis and wherein said antibody fragment:

a) reacts with normal human platelets and with dog blood platelets,

b) fails to react with thrombasthenic human platelets or human platelets whose GPIIb/IIIa complex is disassociated with EDTA and

c) reacts slowly with unactivated platelets and more rapidly with ADP activated platelets,

2. The fragment of a monoclonal antibody of Claim 1, wherein the thrombosis is a thrombotic attack.

3. The fragment of a monoclonal antibody of Claim 1 which is produced from the digestion of a monoclonal antibody produced from a hybridoma formed by the fusion of X63-Ag 8.653 BALB/c murine myeloma cells and spleen cells from the BALB/c mice previously immunized with human blood platelets, with a proteolytic agent selected from the group consisting of papain and pepsin.

4. The fragment of a monoclonal antibody of Claim 3, wherein the proteolytic agent is pepsin.

B

5. A monoclonal antibody fragment which inhibits platelet function, produced by the sequential steps of:

i) immunizing mice with human blood platelets,

ii) removing the spleens from said mice and making a suspension of the said spleen cells,

iii) fusing said spleen cells with mouse myeloma cells in the presence of a fusion promoter,

iv) diluting and culturing the fused cells in separate wells in a medium which will not support the infused myeloma cells,

v) evaluating the supernatant in each well containing a hybridoma for the presence of antibody to the fibrinogen receptor,

vi) selecting and cloning a hybridoma producing antibody that:

a) reacts with normal human platelets and with dog blood platelets,

b) fails to react with thrombasthenic human platelets or human platelets whose GPIIb/IIIa complex is disassociated with EDTA and

c) reacts slowly with unactivated platelets and more rapidly with ADP activated platelets,

vii) recovering the antibody from the supernatant above said clones,

B1

viii) digesting said antibody with a proteolytic agent selected from the group consisting of papain and pepsin,

ix) recovering said fragment from the digestion mixture.

6. The fragment of a monoclonal antibody of Claim 5, wherein the proteolytic agent is pepsin.

7. A monoclonal antibody fragment which inhibits platelet function that reacts more rapidly with ADP activated platelets than unactivated platelets, produced by the sequential steps of:

i) immunizing mice with human blood platelets,

ii) removing the spleens from said mice and making a suspension of the said spleen cells,

iii) fusing said spleen cells with mouse myeloma cells in the presence of a fusion promoter,

iv) diluting and culturing the fused cells in separate wells in a medium which will not support the infused myeloma cells,

v) evaluating the supernatant in each well containing a hybridoma for the presence of antibody to the fibrinogen receptor,

vi) selecting and cloning a hybridoma producing antibody that:

a) reacts with normal human platelets and with dog blood platelets,

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b) fails to react with thrombasthenic human platelets or human platelets whose GPIIb/IIIa complex is disassociated with EDTA and

c) reacts slowly with unactivated platelets and more rapidly with ADP activated platelets,

vii) transferring said clones intraperitoneally into mice,

viii) harvesting the malignant ascites or serum from said mice, which ascites or serum contains the desired antibody,

ix) digesting said antibody with a proteolytic agent selected from the group consisting of papain and pepsin,

ix) recovering said fragment from the digestion mixture.

8. The fragment of a monoclonal antibody of Claim 7, wherein the proteolytic agent is pepsin.

9. A method of preparing a fragment of a monoclonal antibody which inhibits platelet function and thrombosis, which comprises the proteolytic digestion of the 7E3 monoclonal antibody, with a proteolytic agent selected from the group consisting of pepsin and papain.

10. The method of Claim 9, wherein the proteolytic agent is pepsin.

11. The fragment of the monoclonal antibody prepared by the method of Claim 9.

12. The fragment of a monoclonal antibody prepared by the method of Claim 9, wherein the proteolytic agent is pepsin.

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(19)(CA) **CANADIAN PATENT** (12)

(54) Platelet Function Inhibiting Monoclonal Antibody Fragment

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(73) Research Foundation of State University of New York (The) , U.S.A.

(30) (US) U.S.A. 621,378 1985/06/14
(US) U.S.A. 851,711 1986/04/14

(57) 12 Claims

Canada

ABSTRACT OF THE DISCLOSURE

There is provided a novel monoclonal antibody fragment which inhibits platelet function and may be useful in the treatment of thrombotic diseases. The fragment is produced by proteolytic digestion of the 7E3 monoclonal antibody.

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Blood platelets, also known as thrombocytes, function in the human body to provide for the coagulation of blood when the blood supply is exposed to potential loss, i.e., trauma, abrasions, and other hemorrhaging injuries. Platelets rapidly interact with damaged blood vessels and with each other to prevent the escape of blood cells and also facilitate the formation of a blood clot in which they become enmeshed so as to plug up any "leaks" in the vascular system. However, in certain disease states, such as thrombosis, it is desirable and even necessary, to inhibit blood platelet function. This is because the thrombus can either occlude the blood vessel and cause damage to the tissue being supplied with blood from that vessel (for example, myocardial infarction, stroke after vascular surgery, etc) or because the platelets and other thrombotic material can break off from the thrombus and lodge down-stream in blood vessels supplying other parts of the body (for example, venous thrombosis with pulmonary embolism, transient ischemic attacks, amaurosis fugax, strokes from the artificial hearts, etc).

Drugs currently utilized to counteract the platelet function suffer from a lack of potency and/or specificity. Thus, there is a continuing need for new and improved drugs that will inhibit platelet function in diseases characterized by thrombosis.

The present invention concerns a monoclonal antibody fragment which inhibits platelet function. The fragment was produced by the proteolytic digestion of the 7E3 monoclonal antibody, suitably with papain or pepsin. The 7E3 monoclonal antibody was produced in the following manner. Human blood platelets were injected into mice. The mouse spleen was removed and fused with a mouse

myeloma by a modification of the technique of Levy et al., (Curr. Top. Microbiol. Immunol., 81, 164 (1978)). The fused cells were incubated and then further incubated in HAT medium (in which non-fused cells will not grow). The cells were then diluted out and screened in a screening assay for antifibrinogen receptor activity. There was selected one particular clone, designated 7E3 of the class IgG_1 which reacts with normal human blood platelets and with dog blood platelets, fails to react with thrombasthenic platelets or human platelets whose GPIIb/IIIa complex was disassociated with EDTA, reacts slowly with unactivated human platelets and more rapidly with ADP activated human platelets and completely blocks the interaction of fibrinogen with platelets induced by ADP.

The Figures I, II and III respectively show as

a) the plots of the change in platelet-rich plasma optical density one minute after ADP addition to blood previously treated with the $F(ab')_2$ fragments at given previous intervals.

b) a plot of molecules of ^{125}I-7E3 bound per platelet ($X\ 10^{-3}$) at certain time intervals after administration of the fragment.

Figure IV (a-d) is a continuous set of plots for one test dog of aortic blood pressure and circumflex blood flow against time after administration of the $F(ab')_2$ fragments.

Figure V is an optical density/time plot of ADP response pre and post $F(ab')_2$ infusion.

a) Preparation

7E3 Monoclonal antibody was prepared according to the procedures set forth below:

Citrated platelet-rich plasma were prepared in accordance with the method of Coller et al., (Blood, 47, 841 (1976)), suspended in a suitable buffer and mixed with

Freund's complete adjuvent. Injections of between about 1 x 10^8 to 5 x 10^8 washed platelets were injected six times at weekly intervals into a BALB/c mouse intraperitoneally and a seventh similar injection without adjuvant at a similar time interval was given intravenously. Three days later the mouse was killed, the spleen removed, the cells separated and fused with a BALB/c mouse myeloma line in accordance with the method of Levy et al., (supra). In this method, the spleen cells and the myeloma cells in a ratio of 3.9:1 were pelletted together, the pellet suspended in polyethylene glycol (35%) in RPMI 1640 medium whereupon the cells were immediately centrifuged at low velocity. The solution was then diluted to about 25% of its previous concentration with RPMI 1640, the cells resuspended, recentrifuged and the supernatant removed. The supernatant was then incubated in a 5% CO_2 95% air atmosphere in RPMI 1640 medium supplemented with fetal calf serum and thereafter selection made in the usual manner by adding HAT medium and aliquoting into microtiter wells. After two weeks, the supernatant of the wells that showed growth were screened for antifibrinogen receptor activity. The clones obtained by this method were selected for various qualities; one in particular, 7E3 was selected for certain qualities.

The antibody was isolated from the supernatant in the wells or flasks. Alternatively, the hybridomas were injected intraperitoneally into Pristane® pretreated BALB/c rats and the antibodies isolated from the ascitic fluid. The antibody was purified by precipitation with 50% saturated ammonium sulfate, resuspended in between 5 and 10% of the original volume in sodium phosphate buffer and dialyzed against the same buffer. Chromatography on protein A-Sepharose® CL-4B equilibrated with phosphate buffer was carried out, elution was with phosphate buffer followed by decreasing pH 0.1M citrate buffers. 7E3 antibody was eluted after the pH decreased to about 6.0. Protein elution was as monitored by ultra-violet

spectroscopy at 280 nm.

Ouchterlony immuno diffusion analysis against anti-IgG1, IgG2a, IgG2b, IgG3, IgM and IgA sera indicated the exclusive presence of IgG1.

Purified antibody was then dialyzed overnight at at reduced temperatures, suitably between about 0 and 10°C preferrably at 4°C against a slightly acidic saline buffer of pH 3.5-6.5, suitably about pH 4.0, after which the freshly prepared pepsin was added in an amount equal to approximately 2% of the antibody's weight. The resulting solution was then incubated at about 37°C for 12 to 24 hours. Digestion was stopped by dialyzing the solution against PBS, pH 7.4. Analysis by polyacrylamide gel electrophoresis indicated that the digestion was essentially complete.

Under somewhat different conditions, an Fab fragment can be prepared by digestion with papain, another proteolytic agent, namely, .1M acetate, 2mM EDTA, 1mM cysteine and including 1% w/w of papain for 6-8 hours at 37° at a pH of 4.5-6, suitably 5.5.

The resulting $F(ab')_2$ fragment of the 7E3 monoclonal antibody can then be further purified by chromatography on a material such as protein A Sepharose CL-4B or DE-52 in order to be certain that any remaining traces of the whole 7E3 monoclonal antibody are removed.

EXAMPLE I

Antibody Production

A BALB/c mouse (Jackson Laboratories, Bar Harbor, Me.) was injected intraperitoneally with six weekly 0.2 ml injections of 3×10^8 washed platelets (citrated PRP washed twice in 0.15 M NaCl, 10 mM Tris/Cl, 10 mM EDTA, pH 7.4 [TS-E]), resuspended in 1/10 to 1/20 of their original volume in TS-E, and mixed 1:1 with complete Freund's adjuvant. The seventh weekly injection was given intravenously into the tail vein and consisted of 0.3 ml. containing 5×10^8 washed platelets resuspended in T-S without EDTA. Each of the seven platelet suspensions was obtained from a different donor. Three days after the last injection, the mouse was killed by cervical dislocation and the spleen removed. A suspension of spleen cells in RPMI 1640 was prepared by teasing the spleen apart. After erythrocytes were lysed with ammonium chloride, the spleen cells were fused with a nonsecretory BALB/c mouse myeloma cel line (X63-Ag 8.653) that had been kept frozen in 10% DMSO, 90% fetal calf serum until one week before fusion, when it was thawed and maintained in the culture medium routinely used (RPMI 1640 supplemental with 10% fetal calf serum and 1,000 U of penicillin and 100 ug of streptomycin/ml). Fusion was carried out according to a modification of the method of Levy et al. (supra). Briefly, 2.7×10^8 spleen cells and 7×10^7 myeloma cells were pelleted together, the pellet was gently suspended in 2 ml of 35% polyethylene glycol in RPMI 1640 medium and the cells immediately centrifuged at 500 g at 22°C for 6 minutes. The solution was then diluted with RPMI 1640 to 9% polyethylene glycol, the cells resuspended and immediately centrifuged at 230 g. for 6 minutes at 22°C. The supernatant fluid was then

aspirated and the fused cells suspended in RPMI 1640 medium and supplemented with 20% fetal calf serum and 10% 109 medium (National Collection of Type Cultures). The cells were placed in a flask and incubated overnight at 37°C in a 5% CO_2, 95% air atmosphere. The following day, the medium was made selective for successfully hybridized cells by adding hypoxanthine (10^{-4} M), aminopterin (4 x 10^{-7} M), and thymidine (1.6 x 10^{-5} M), after which the cells were aliquoted into 960 microtiter wells (Costar, Data Packaging, Cambridge, Ma.). Two weeks later, 574 wells showed growth and the supernatant fluids from 59 wells were positive in a screening assay for antifibrinogen receptor activity (see below). After an additional two weeks in culture, the positive clones were transferred to 24-well microtiter dishes (Costar) and fed with the same medium as above, but without the aminopterin. The clones were expanded and the cells that continued to produce antifibrinogen receptor antibody were suspended in 90% fetal calf serum-10% DMSO and frozen in liquid nitrogen.

The clones were subcloned by both limiting dilution technique and growth in soft agar to insure monoclonality.

Ascitic fluid rich in 7E3 antibody was prepared by intraperitoneal injection of Pristane-pretreated BALB/c mice with 5 x 10^6 hybrid cells that had been washed twice in 0.15 M NaCl, 10 mM sodium phosphate, pH 7.4 (PBS).

EXAMPLE II

Screening Assay

35 ul of PRP (platelet rich plasma) (adjusted to 3 x 10^{11} platelets/liter) and 35 ul. of the supernatant culture medium (or ascitic fluid) to be assayed were incubated together for 2-60 minutes in a well of a round-bottomed microtiter plate (Linbro Chemical Co., Hamden, Ct). 5 ul of the fibrinogen-coated bead suspension was

then added and the plate was mixed on a rotator (Tekator V, American Scientific Products, Edison, N.J.) for 5 minutes at 280 rpm. The wells were observed from the bottom with the aid of a magnifying mirror apparatus (Cooke Microtiter System, Dynatech Laboratories, Inc., Alexandria, Va.). Wells containing culture medium that had not been used for growing cells showed marked agglutination of the beads (rated as 4+), whereas the supernatant culture medium or mouse ascitic fluid from positive clones inhibited the agglutination, resulting in lower readings (0-3+).

EXAMPLE III

Antibody Purification

Culture supernatants were precipitated at 4°C with 50% saturated ammonium sulfate and resuspended to between 1/20 and 1/10 of their original volume in 0.1 M sodium phosphate buffer, pH 8.0. After dialysis against the same buffer, the samples were applied to a 0.8 x 15.9 cm column of protein A Sepharose CL-4B that had been equilibrated with the phosphate buffer (after having been washed with both the phosphate buffer and a 0.1 M citrate buffer, pH 3.0). The column was eluted with the phosphate buffer until the optical density of the eluate returned to base line, after which stepwise elution was accomplished with 0.1 M citrate buffers of pH 6.0, 4.5, 3.5 and 3.0, as described by Ey, et al., (Immunochemistry, 15, 429 (1978). 7E3 immunoglobins were eluted at pH 6.0. Protein elution was monitored by optical density at 280 nm and appropriate fractions were pooled and dialyzed against T-S containing 0.05% sodium azide. Antibody concentration was estimated by absorption at 280 nm, assuming $A^{1\%} = 15$.

EXAMPLE IV

Preparation of Fragment

7E3 Monoclonal antibody was prepared according to the

procedures disclosed in the foregoing examples and the hybridoma 7E3 has been deposited at the American Type Culture Collection under the accession number HB8832.

Purified antibody was then dialyzed overnight at approximately 4°C against 0.2M sodium chloride, 0.2M acetate, pH 4.0, after which freshly prepared pepsin (1 mg/ml) was added in an amount equal to approximately 2% of the antibody's weight. The resulting solution was then incubated at about 37°C for 12 to 24 hours. Digestion was stopped by dialyzing the solution against PBS, pH 7.4. Analysis by polyacrylamide gel electrophoresis indicates that the digestion was essentially complete.

The resulting $F(ab')_2$ fragment of the 7E3 monoclonal antibody can then be further purified by chromatography on a material such as protein A Sepharose CL-4B or DE-52 in order to be certain that any remaining traces of the whole 7E3 monoclonal antibody are removed.

EXAMPLE V

Testing

The $F(ab')_2$ monoclonal antibody fragment was then tested for platelet function inhibition by the following method:

Three dogs were utilized. Dog No. 1 received a total of 0.17 mg/kg. in three injections given one hour apart. A blood sample drawn one hour after the last injection showed the following: 1) had a 61% decrease in the maximum extent of aggregation (Tmax) induced by 4 mADP; 2) having a platelet count of 220,000/ul (control (t = 0)) = 236,000; and 3) bound 45,400 molecules/platelets of ^{125}I-7E3 (control = 56,400), which suggests that 11,000 molecules of the $F(ab')_2$ fragment of the 7E3 monoclonal antibody are bound per platelet. Dog No. 2 received a single 0.57 mg/kg dose. 1.5 Hours later, the Tmax to ADP had decreased by 68% and the number of molecules of ^{125}I-7E3 bound/platelet had decreased by 59% (from 62,500 to 25,400); at 4 hours there was an 84% reduction in Tmax and

a 64% reduction in molecules bound (22,500). The initial platelet count of 265,000 decreased to 253,000 at 1.5 hours and to 213,000 at 4 hours, but returned to 242,000 at 20 hours. Neither dog bled spontaneously.

A third dog received 0.8 ug/kg of fragment as shown in Figure III. These test results indicate that the $F(ab')_2$ fragment of the 7E3 monoclonal antibody can dramatically inhibit platelet function in vivo without causing profound thrombocytopenia.

It will be apparent to those skilled in the art that many modifications, both of materials and methods, may be practiced without departing from the purpose and intent of this disclosure.

EXAMPLE VI

In Vivo Testing

The cyclical blood flow reduction model has previously been described in detail (Folts, J.D., E.B. Crowell, Jr., and G.G. Rowe, 1976, Platelet Aggregation in Partially Obstructed Vessels and its Elimination with Aspirin, Circulation 54:365-370; Folts, J.D., 1982, Experimental Arterial Platelet Thrombosis, Platelet Inhibitors and their Possible Clinical Relevance, Cardiovasc. Rev. Rep., 3:370-382; Folts, J.D., Gallagher, K.., and Rowe, G. G., 1982, Blood Flow Reductions in Stenosed Canine Cornary Arteries: Vasopasm or Platelet Aggregation, Circulation 65:248-255). Briefly, either the circumflex coronary artery of an anesthetized dog or the carotid artery of an anesthetized monkey is dissected out and an electromagnetic probe is placed on it to measure blood flow. The artery is stenosed approximately 70% by placing a 3-4 mm long encircling plastic cylinder of the appropriate internal diameter

around the outside of the artery. Cyclical reductions in blood flow due to platelet thrombus formation then commence whether spontaneously or after additional intimal damage is produced by brief (ca. 1 sec.) clamping of the artery with a hemostat. Within several minutes, blood flow in the vessel decreases to a critical level, after which the flow is abruptly restored to its original level, either by spontaneous embolization of the platelet thrombus, or by embolization induced by gently shaking the cylinder. Once established, the cyclical flow reductions persist if no intervention is made. The frequency and amplitude of the flow reductions can be temporarily enhanced by infusing 0.5 ug/kg/min. of epinephrine intravenously for 10 min.

The experimental protocol consisted of first obtaining samples of blood anticoagulated with either 0.01 volume 40% sodium citrate (for platelet aggregation and ^{125}I-7E3 binding), or 0.037 volume 269 mM EDTA (for platelet counts), and then performing the surgery. After cyclical flow reductions were established, another set of blood samples was obtained and the antibody (0.7-0.8 mg/kg) was infused intravenously as a bolus. A final set of samples was obtained 30 min. after infusing the antibody. Platelet counts on the platelet-rich plasma were performed by microscopy after dilution and lysis of erythrocytes (Unopette, Becton-Dickinson), and whole blood platelet counts were performed by an electronic resistive particle counter (S+IV, Coulter Electronics, Hialeah, FL.).

Results

Cyclical reductions in blood flow were obtained in 4 dogs and 4 monkeys. In all 4 dogs and 4 monkeys, infustion of the $F(ab')_2$ fragments resulted in complete cessation of the cyclical flow reductions and restoration of the control flow rate in 10 min. or less (Figure IV). In the dogs, restoration of coronary flow correlated with the disappearance of ST segment deviations on the electro-

cardiograms, indicating the reversal of myocardial ischemia. The cyclical flow reductions could not be restored with epinephrine infusions of 0.5 or 1 ug/kg/min. for 10 minutes, increasing the intimal damage by repeated brief clamping of the vessel with a hemostat, passing current (1-2 mA) through the cylinder, or a combination of these provocations (Figure IV). Postmortem histologic examination of the blood vessels confirmed the presence of extensive intimal damage.

Platelet aggregation in response to ADP (≤25 uM) was virtually abolished by the antibody infusion in all 8 animals, although the shape change response remained intact (Figure V). ^{125}I-7E3 antibody binding studies were performed on 1 dog and 1 monkey. In both animals 84% of the GPIIb/IIIa receptors were blocked by the F(ab')$_2$ fragments (dog = 34,300 molecules of ^{125}I-7E3 bound per platelet before infusion and 5,500 after; monkey = 49,700 before and 8,100 after). Platelet counts decreased by less than 15% in the 3 animals tested. None of the animals demonstrated a significant change in heart rate or blood pressure upon antibody infusion. There was no clear evidence of excessive hemorrhage from the operative sites after antibody infusion, although no objective data on blood loss were obtained.

DOG 1
(0.17 mg/kg

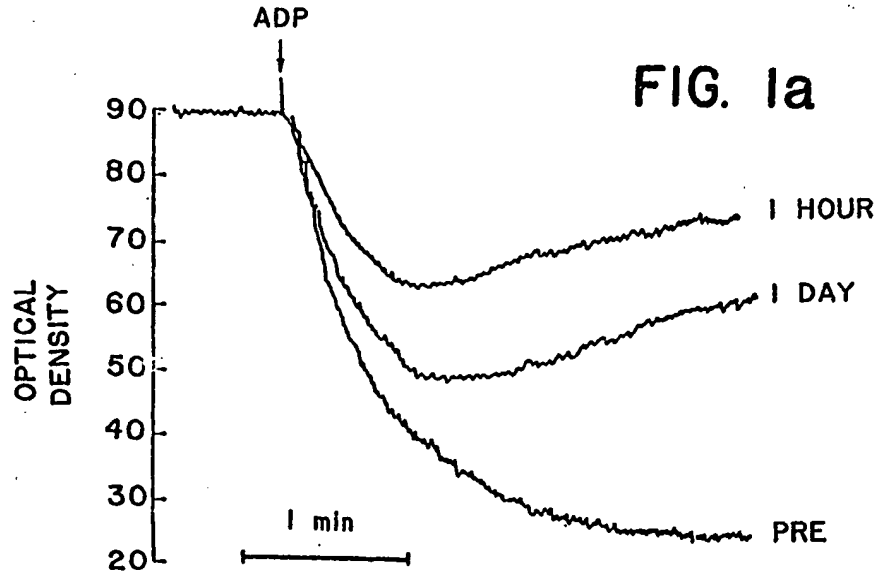

FIG. 1a

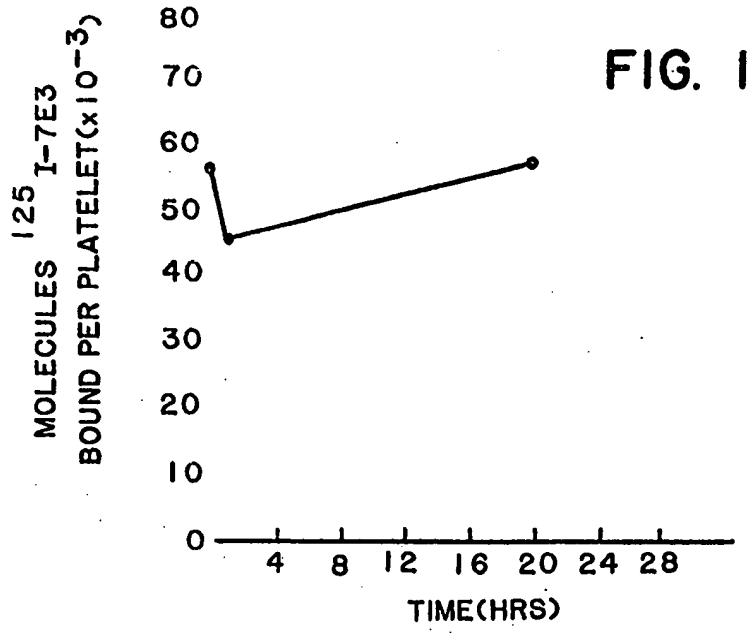

FIG. 1b

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6-2

DOG 2

(0.57 mg/kg)

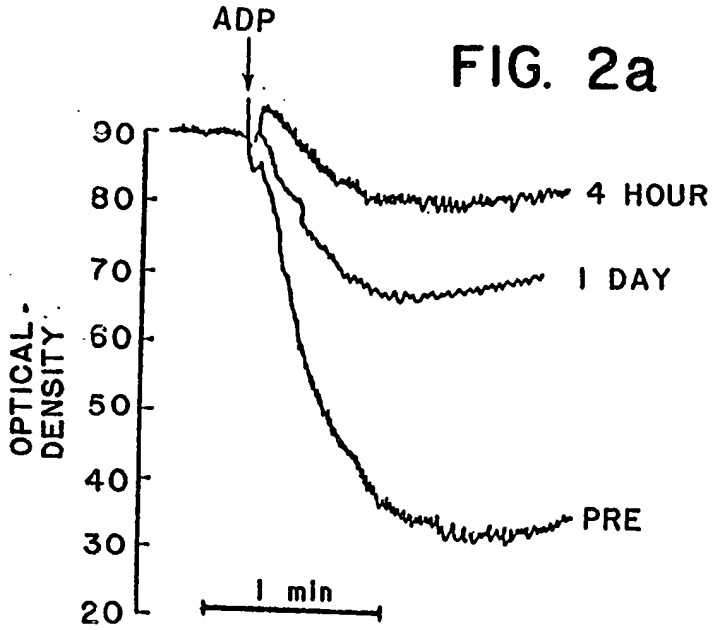

FIG. 2a

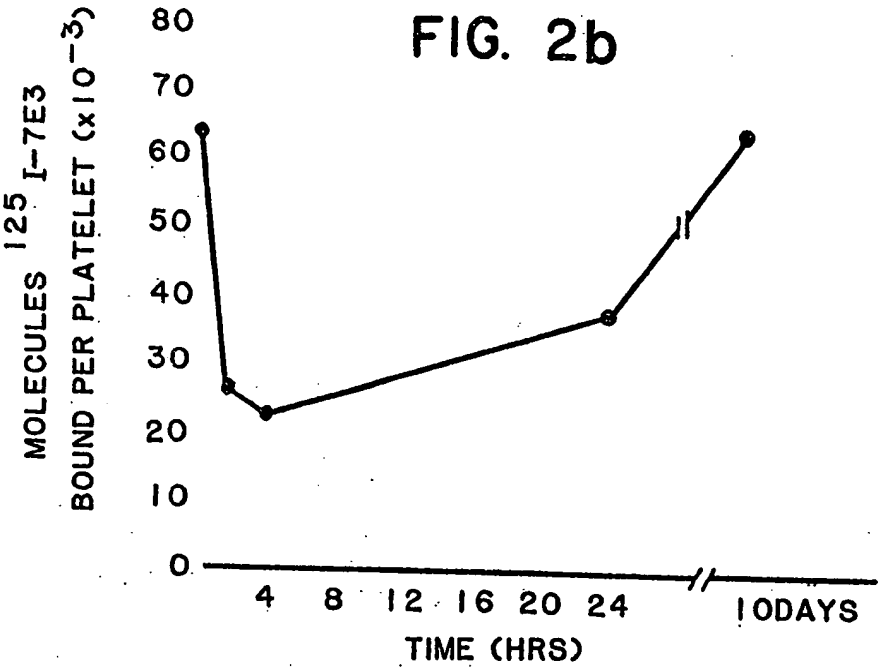

FIG. 2b

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DOG 3

(0.81 mg/ml)

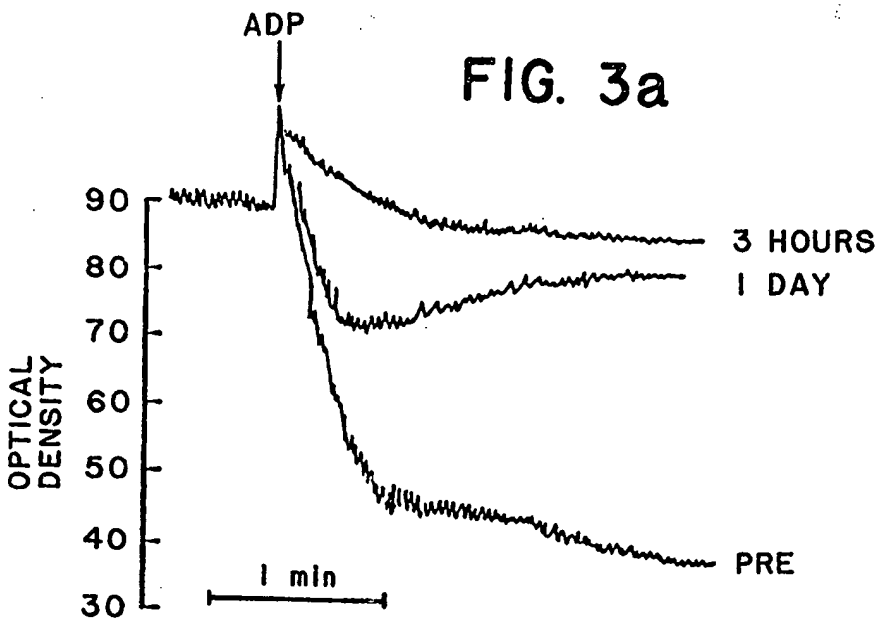

FIG. 3a

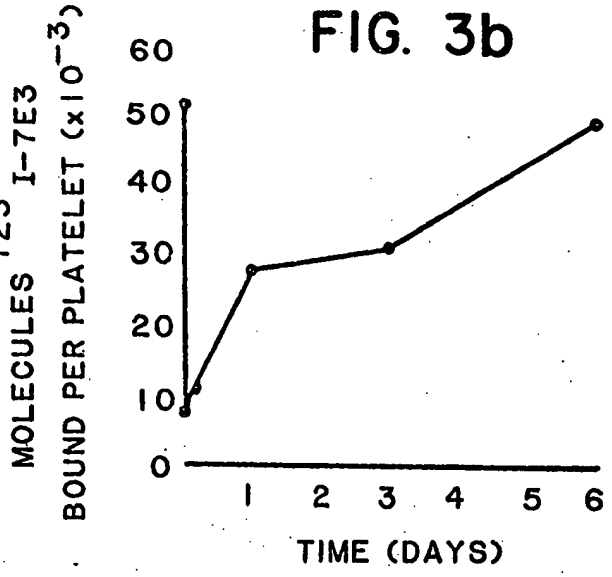

FIG. 3b

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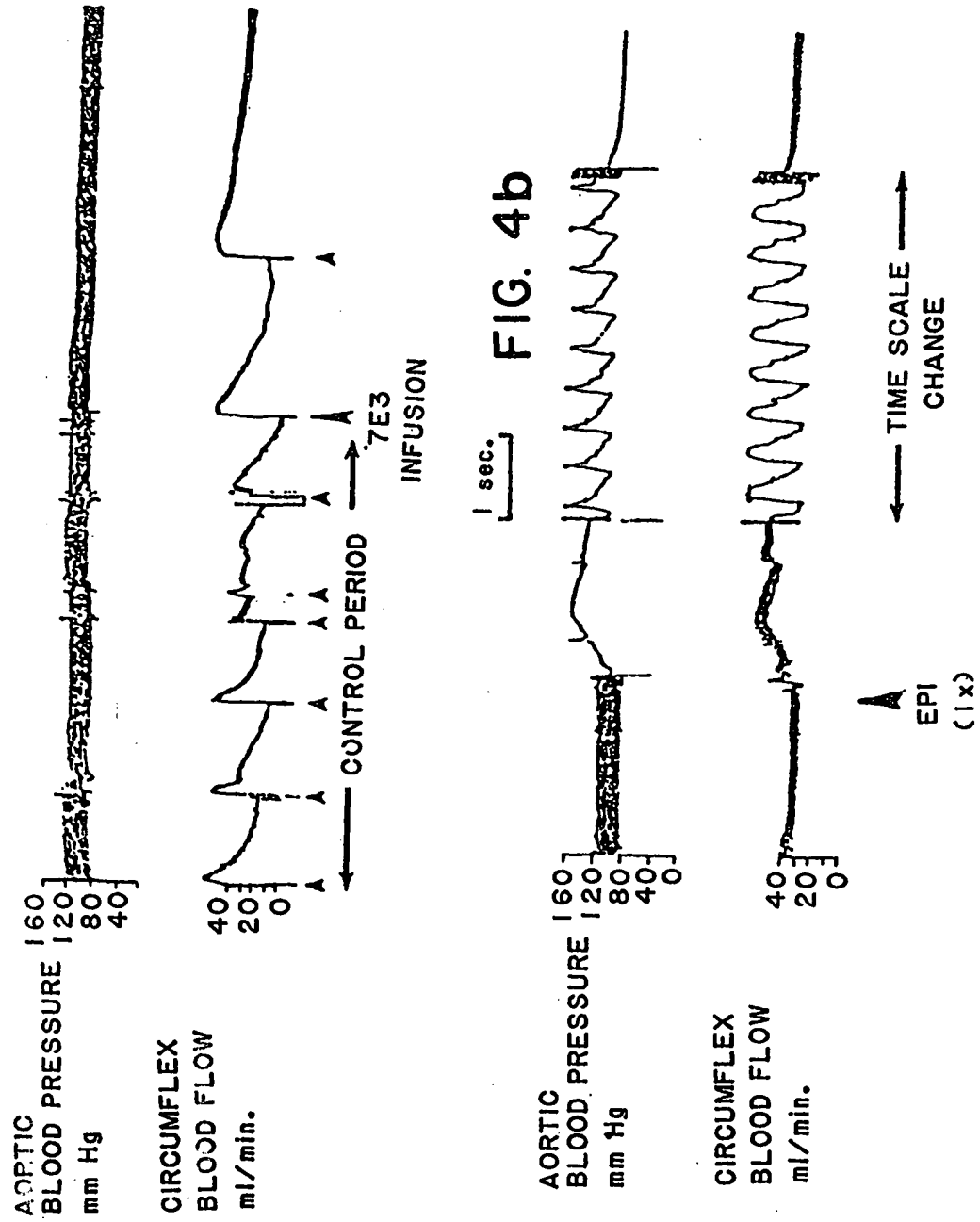

FIG. 4a

FIG. 4b

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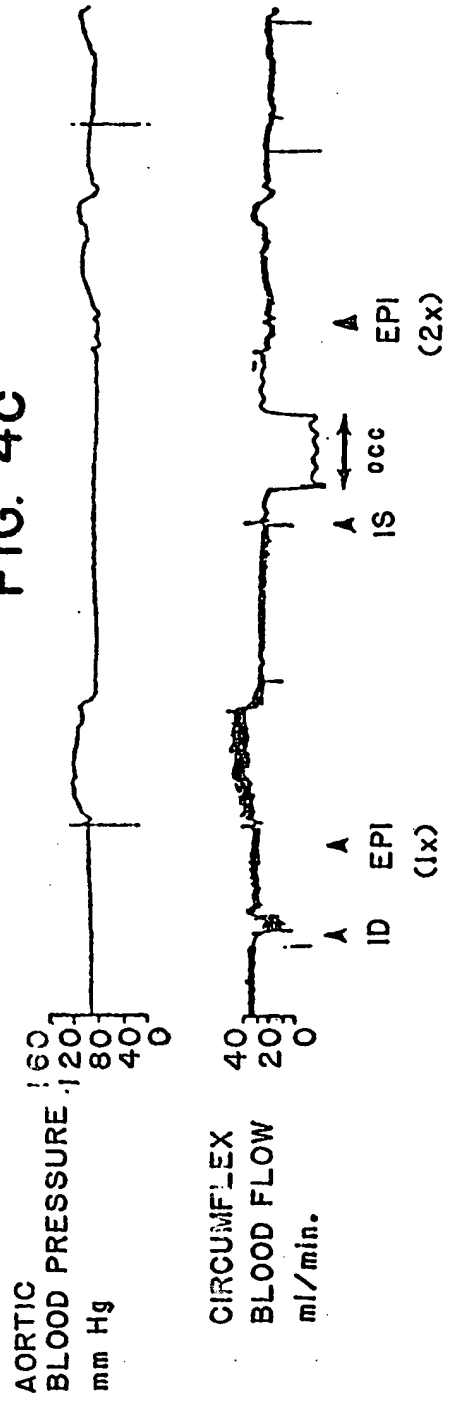

FIG. 4c

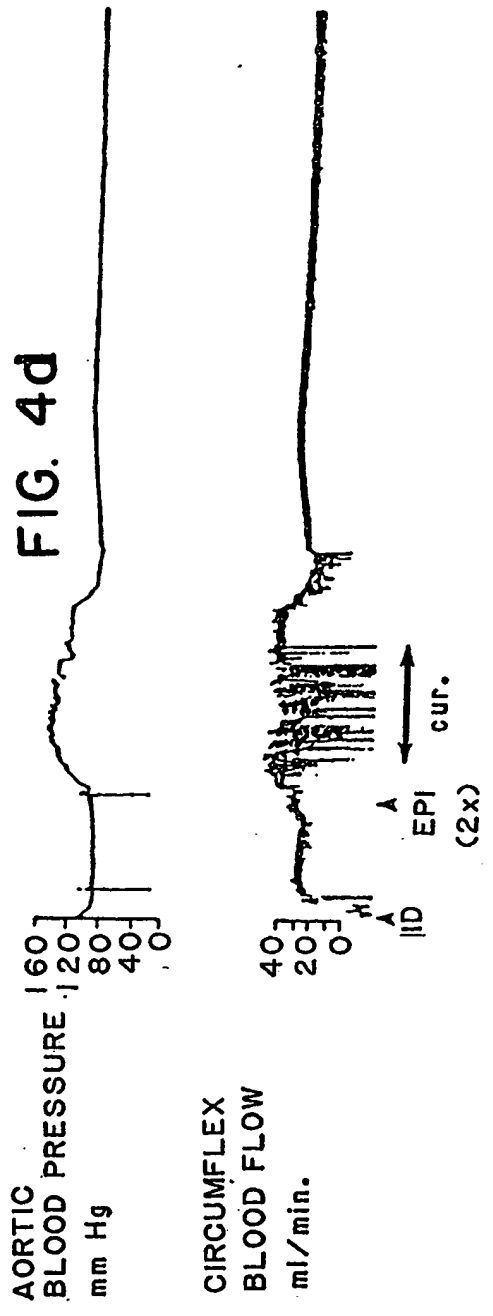

FIG. 4d

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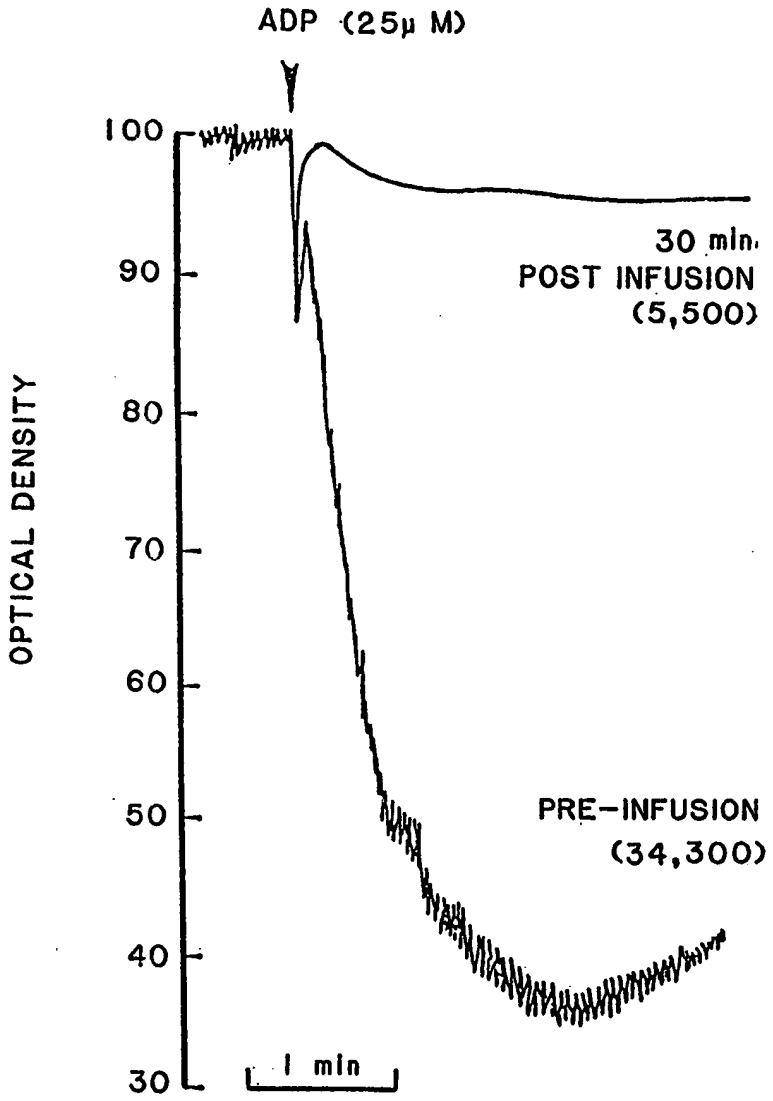
FIG. 5



Andreas Hage Debus & Matthew Walker